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Deciphering carbon sources of mussel shell carbonate under experimental ocean acidification and warming

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ABSTRACT

Ocean acidification and warming is widely reported to affect the ability of marine bivalves to calcify, but little is known about the underlying mechanisms. In particular, the response of their calcifying fluid carbonate chemistry to changing seawater carbonate chemistry remains poorly understood. The present study deciphers sources of the dissolved inorganic carbon (DIC) in the calcifying fluid of the blue mussel (*Mytilus edulis*) reared at two pH (8.1 and 7.7) and temperature (16 and 22 °C) levels for five weeks. Stable carbon isotopic ratios of seawater DIC, mussel soft tissues and shells were measured to determine the relative contribution of seawater DIC and metabolically generated carbon to the internal calcifying DIC pool. At pH 8.1, the percentage of seawater DIC synthesized into shell carbonate decreases slightly from 83.8% to 80.3% as temperature increases from 16 to 22 °C. Under acidified conditions, estimates of percent seawater DIC incorporation decreases clearly to 65.6% at 16 °C and to 62.3% at 22 °C, respectively. These findings indicate that ongoing ocean acidification and warming may interfere with the calcification physiology of *M. edulis* through interfering with its ability to efficiently extract seawater DIC to the calcifying front.

1. Introduction

Approximately 40% of anthropogenically-derived carbon dioxide (CO_2) is known to eventually enter the marine realm, consequently leading to ocean warming and acidification (IPCC, 2014). Rapid uptake of anthropogenic CO_2 by the ocean has profoundly changed its carbonate system, in particular decreasing the availability of carbonate (CO_3^{2-}) ions and corresponding saturation state of calcium carbonate (Ω) in seawater (Waldbusser and Salisbury, 2014). As a result, many studies of marine calcifying species, especially bivalve mollusks, have demonstrated negative impacts of changing seawater chemistry on their abilities to build shells and/or skeletons (e.g., Hendriks et al., 2010; Gazeau et al., 2013; Kroeker et al., 2013; Bach, 2015; Fassbender et al., 2016). With increasing seawater temperature and pCO_2, for example, many bivalves are found to grow slower, take longer to develop and suffer higher mortality (e.g., Talmage and Gobler, 2010; Dickinson et al., 2012; Scanes et al., 2014; Fitzer et al., 2015; Milano et al., 2016; Sui et al., 2016; Xu et al., 2016; Zhao et al., 2017a). Notably, these consequences occurring at one life-history stage may generate carry-over effects on their subsequent life-history stages or even next generation (e.g., Hettinger et al., 2012; Gobler and Talmage, 2013; Fitzer et al., 2014; Parker et al., 2012, 2015; Griffith and Gobler, 2017; Thomsen et al., 2017). Furthermore, alternations in seawater chemistry resulted from CO_2 uptake can affect the recruitment success and thereby population maintenance of marine bivalves (Hendriks et al., 2015). As many bivalves are ecologically critical foundation species in coastal waters, profound effects of ocean acidification on bivalves may eventually result in serious ecological consequences (Gobler and Talmage, 2013).

To accurately predict how marine bivalves respond in the context of global climate change, it is imperative to elucidate the driving mechanisms of widely observed reductions in both shell calcification and growth (for a detailed review, see Gazeau et al., 2013). Following larval settlement and metamorphosis, juvenile and adult individuals start to precipitate their shells in the extrapallial space (EPS), where the rate of calcium carbonate ($CaCO_3$) deposition is dependent on the Ω state at the site of calcification. Subsequently, crystallization, which is orchestrated by the organic matrix, is synthesized from the outer mantle epithelium (Marin et al., 2012). Meanwhile, end calcification products (i.e., H^+ ions) are actively pumped out the EPS via H^+-ATPase, Ca^{2+}-ATPase and/or Na^+/H^+ exchanger (Li et al., 2016; Zhao et al., 2017b). Under acidified conditions, decreases of seawater pH and CO_3^{2-}

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availability can likely hinder the ability of the bivalves to maintain the Ω state and pH homeostasis in the EPS, consequently resulting in slower rates of shell growth (Waldbusser et al., 2015; Thomsen et al., 2015; Ramesh et al., 2017). Likewise, given the energetically expensive nature of organic matrix synthesis, which may account for 10–60% of energetic requirement for somatic growth and 15–150% during gonadal maturation (Palmer, 1992), reductions in the synthesis and secretion of organic matrix for shell mineralization can serve as an alternative mechanistic explanation (e.g., Hüning et al., 2013; Ramajo et al., 2016).

Despite previous advances in understanding the combined effects of ocean acidification and warming on molluscan shell formation, a substantial gap in our knowledge remains about sources of dissolved inorganic carbon (DIC) in the EPS used for calcification. Such information is of paramount importance for advancing the currently proposed, albeit hotly debated, hypotheses, i.e., the omega or proton myth. The omega hypothesis proposes the sensitivity of molluscan calcification to the calcium carbonate saturation state (e.g., Waldbusser et al., 2015, 2016), while accumulating evidence suggests that reduced calcification rates in an acidifying ocean are driven by increases of seawater H^+ concentration (e.g., Roleda et al., 2012; Cyronak et al., 2015, 2016). Yet, it is worth noting that both hypotheses stem from the assumption that bivalves exclusively or mainly transport the DIC from the seawater to calcify. Furthermore, given that the total DIC pool of seawater consists of three inorganic carbon species (i.e., CO_2, HCO_3^- and CO_3^{2-}), it is important to elucidate which species is closely relevant for calcification. Such information is critical to better understand the calcification response of marine bivalves to CO_2-driven changes in seawater carbonate chemistry. More specifically, it is generally assumed that HCO_3^- in seawater is the most calcification-relevant carbon species (Bach, 2015), as it contributes approximately 92–95% to the total DIC pool (Beirne et al., 2012). For instance, as schematically illustrated in Fig. 1, two molecular HCO_3^- transporters, i.e., Na^+/HCO_3^- transporter and HCO_3^-/Cl^- exchanger, have been localized in the outer mantle epithelium. CO_3^{2-} is the most suitable substrate for inorganic $CaCO_3$ precipitation (Romanek et al., 1992). However, no molecular systems responsible for CO_3^{2-} transport have been described in bivalve mollusks. The mantle epithelium is naturally permeable to CO_2 (Gutknecht et al., 1977). Therefore, it is also likely that seawater CO_2 can easily diffuse into the EPS. In particular, CO_2 is rapidly catalyzed by the carbonic anhydrase into HCO_3^- during the active calcification, consequently sustaining a large pCO_2 gradient between the EPS and the ambient seawater (Fig. 1). Taken together, it is reasonable to assume that HCO_3^- and CO_2 are the more suitable calcification-relevant DIC species taken up by marine bivalves to calcify.

Recent works, however, have demonstrated that shell carbonate is synthesized not only from seawater DIC but also metabolically generated CO_2, with the relative contribution of the latter spanning 5% up to 37% (e.g., Gillikin et al., 2007; Poulain et al., 2010; Beirne et al., 2012; Marchais et al., 2015). In particular, it is well known that elevated seawater pCO_2 can constrain the passive diffusion of metabolic CO_2 (Zittier et al., 2015), resulting in its retention and subsequent incorporation into the EPS. Notably, as seawater temperature and pCO_2 increase, bivalves often increase their metabolic activities (reviewed by Gazeau et al., 2013), which, in turn, increase the amount of metabolically generated CO_2 transported into the EPS. Quantitative determination of the relative contribution of metabolic carbon to shell carbonate, therefore, represents a critical step toward elucidating how CO_2-driven changes in seawater carbonate chemistry affect the ability of marine bivalves to build their shells (Bach, 2015).

The present study aims to investigate whether, and to what extent, carbon sources of shell carbonate diversify in the face of experimental scenarios of ocean acidification and warming. The blue mussel, *Mytilus edulis*, is selected as the model species, considering that the fate of this ecologically and economically important molluscan species in the context of global climate change has received much attention during the past decade (Gazeau et al., 2013). The relative contribution of seawater DIC and metabolically generated carbon synthesized into mussel shell carbonate is determined by stable carbon isotopic analysis.

2. Materials and methods

2.1. Bivalve collection and acclimation

Specimens of adult *M. edulis* (24–32 mm shell length) were collected from Xinghai Bay, Yellow Sea (38°52′34.51″ N, 121°33′42.47″ E). Water temperature, salinity and pH at the sampling site were 15.6 °C, 31 PSU and 8.02, respectively. Upon arrival in the laboratory (within 20 min), mussels were maintained in a 500 L tank with circulating

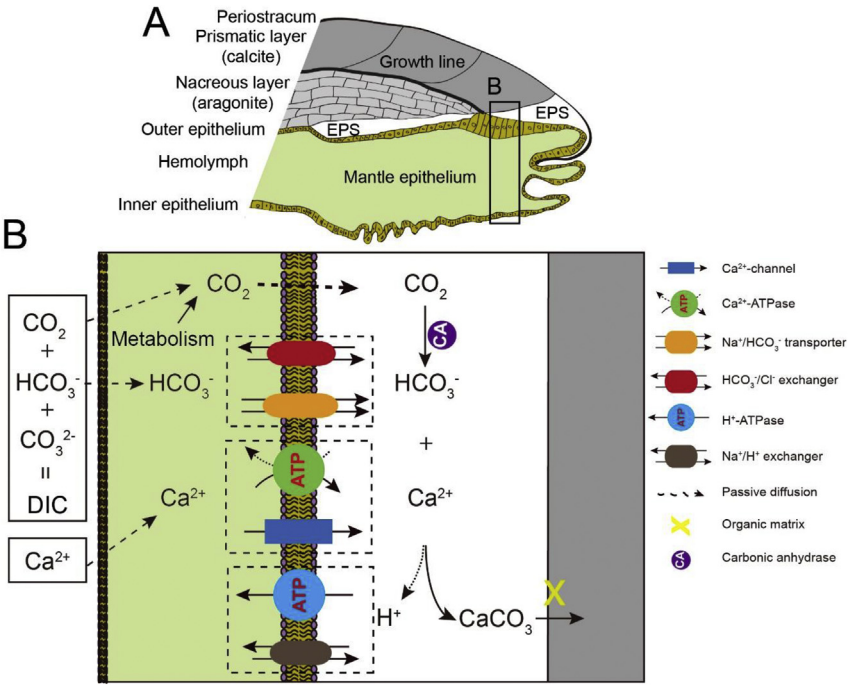

Fig. 1. Conceptual model of mussel shell formation. (a) Schematic section through the mussel showing major shell structures. EPS: extrapallial space. (b) Underlying mechanisms involved in shell formation. *Mytilus edulis* is an osmoconformer organism able to maintain its hemolymph chemically similar to the ambient seawater. Therefore, calcification-relevant substrates (Ca^{2+} and DIC) are able to easily across the inner mantle epithelium to the hemolymph, while these ions are actively transported with the aid of ionic transporters in the outer mantle epithelium to the EPS. For example, Ca^{2+} channel and Ca^{2+}-ATPase are responsible for Ca^{2+} transport, and Na^+/HCO_3^- transporter and HCO_3^-/Cl^- exchanger are involved in the HCO_3^- transport, while seawater and metabolically generated CO_2 can passively diffuse into the EPS where it is quickly catalyzed into HCO_3^- by the carbonic anhydrase. In the EPS, inorganic $CaCO_3$ precipitates via the reaction $Ca^{2+} + HCO_3^- \rightarrow CaCO_3$. The crystallization of $CaCO_3$ is controlled by the organic matrix synthesized and secreted from the outer mantle epithelium. Protons generated during the $CaCO_3$ precipitation are pumped out via H^+-ATPase, Ca^{2+}-ATPase and/or Na^+/H^+ exchanger. Modified from Zhao et al. (2018b).

seawater for a two-week acclimation period, during which they were fed daily with a 1:1 mixture of two microalgal strains (*Nitzschia closterium* and *Platymonas subcordiformis*). Environmental conditions (16 °C, 31 PSU and pH 8.0–8.1) were rather comparable to those recorded at the sampling site and day of collection. No mortality was observed throughout this period.

2.2. Experimental setup

To evaluate the combined effects of ocean warming and acidification, four identical recirculating systems, which correspond to the combinations of seawater temperature (16 and 22 °C) and pH (7.7 and 8.1), were established, as schematically illustrated in Xu et al. (2016). In brief, each system comprised three experimental tanks (120 L per tank), one temperature controlling tank (120 L) and one gas mixing tank (120 L), holding a water column of 600 L in total. It should be emphasized here that, according to Cornwall and Hurd (2015), this experimental design is categorized into a randomized design with interdependent-replicates (i.e., pseudo-replicates). The temperature of 16 °C corresponds to the sea surface temperature, while 22 °C corresponds to an upper-end warming scenario of approximately 5.1 °C by the year 2100 (Sokolov et al., 2009). Likewise, pH 7.7 mimics near-future ocean acidification projected for the year 2100 (IPCC, 2014), which was achieved by bubbling pure CO_2 gas into the gas mixing tank mentioned above. It is also worth noting here that, throughout the following texts and figures, reference is made to above nominal temperature and pH levels rather than measured values, as throughout the experiment seawater temperature, salinity and pH levels were kept stable.

To identify the newly deposited shell portion during the experiment, mussels were immersed to calcein (150 mg L^{-1}) for 3 h. As a fluorescent dye, calcein is not toxic and it is immediately incorporate into the crystal lattice of the shell (Thébault et al., 2006). Under a UV-light microscope, calcein fluoresces bright green (Kaehler and McQuaid, 1999). After staining, 20 mussels were randomly assigned to each experimental tank. During the following five weeks, they were reared under conditions previously designated. To maintain water quality, every two days half of the water in each tank was renewed. Mussels were fed every two days with an equal mixture of *N. closterium* and *P. subcordiformis* at a concentration of circa 40,000 cells mL^{-1}. Temperature, salinity and pH were monitored daily. On a weekly basis, water samples for total alkalinity (TA) and stable carbon isotopic analyses were filtered using Whatman GF/F filters, poisoned using 1 mg l^{-1} $CuCl_2$ to stop microbial activities and stored at 4 °C for further analysis. The CO2SYS software program (Pierrot et al., 2006) was used to calculate the major seawater carbonate system parameters (for details see Zhao et al., 2017a).

2.3. Sample preparation

Six mussels from each treatment (i.e., two mussels from each replicate for each treatment) were randomly selected for isotopic analysis. Each specimen was carefully dissected using a plastic scalpel, and the soft tissue and shell were rinsed with tap water and dried at 50 °C for 48 h in an oven. Whole soft tissue was subsequently homogenized. The right valve of each shell was mounted on a Plexiglass cube with a quick-drying EpoFix plastic welder, and to prevent shell breakage, the axis of maximum growth was coated with a layer of JB KWIK epoxy resin (Fig. 2). A three-millimeter-thick section was cut using a low-speed precision saw. Each shell slab was mounted on a glass side, ground with 800 and 1200 grit SiC, polished with 1 μm Al_2O_3 and ultrasonically rinsed with deionized water. Shell slabs were examined under a fluorescent light microscope to locate the calcein marks, and the entire newly deposited prismatic portions (i.e., increments between the calcein mark and ventral margin; Fig. 2) were drilled to obtain shell carbonate powder.

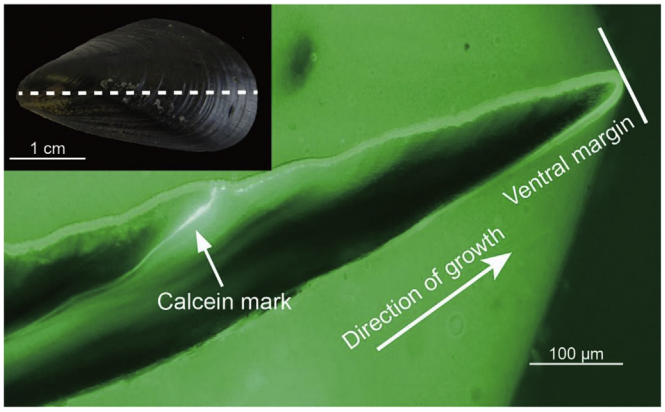

Fig. 2. Cross section of *Mytilus edulis shell* showing the calcein mark. The while dotted line suggests the axis of maximum shell growth along which a three-millimeter-thick shell slab was cut.

2.4. Carbon isotopic analysis via EA-IRMS and CF-IRMS

Approximately 1–2 mg soft tissue powder was weighted into a tin capsule, packed and measured with a Thermo Quest Delta plus XL isotope ratio mass spectrometer (IRMS) coupled to a Flash 1112 elemental analyzer (EA). Water samples (10 mL) were acidified with 200 μL concentrated orthophosphoric acid overnight and then measured with the IRMS. Repeated analysis of several standard materials obtained a precision better than 0.2‰. Measured carbon isotopic ratios were calculated against the Vienna Peedee Belemnite (VPDB) and expressed in the standard delta (δ) notation in per mil (‰).

Approximately 40–100 μg shell carbonate powder was weighted into a glass vial, dissolved with concentrated phosphoric acid at 72 °C for 2 h in He-flushed borosilicate exetainers. The liberated CO_2 gas was measured with a Thermo Finnigan MAT 253 continuous flow–isotope ratio mass spectrometer (CF-IRMS) coupled with a GasBench II automated carbonate reaction device. Measured isotopic data were calibrated against NBS-19, yielding an average accuracy better than 0.04‰. Carbonate carbon isotopic ratios were calculated and expressed in the same manner as the mantle tissue and seawater.

2.5. Data analysis

The $\delta^{13}C$ of bivalve shells is controlled by ambient seawater $\delta^{13}C_{DIC}$, the $\delta^{13}C$ of metabolic CO_2, as well as the relative proportion of seawater DIC and metabolic carbon incorporated into shell carbonate (McConnaughey and Gillikin, 2008). Hence, carbon isotopic analysis can be used to delineate carbon sources of *M. edulis* shell carbonate. Quantitative determination of the relative contribution of metabolic carbon can be calculated using the following equation described by McConnaughey et al. (1997).

$$\%C_M = \frac{\delta^{13}C_{shell} - \varepsilon_{cal-b} - \delta^{13}C_{DIC}}{\delta^{13}C_M - \delta^{13}C_{DIC}} \times 100 \quad (1)$$

where $\%C_M$ is the percentage of metabolic carbon synthesized into shell carbonate. $\delta^{13}C_{shell}$, $\delta^{13}C_{DIC}$ and $\delta^{13}C_M$ represent the $\delta^{13}C$ of shell carbonate, ambient seawater DIC and metabolic CO_2, respectively, and ε_{cal-b} represents the enrichment factor between calcite and biocarbonate (1‰ calculated by Romanek et al., 1992). In the present study, the $\delta^{13}C$ of the soft tissue was used to approximate $\delta^{13}C_M$ according to McConnaughey et al. (1997).

Experimental data were statistically analyzed using the software SPSS (version of 19.0). Prior to analysis, the normality of data and homogeneity of variance were examined using the Shapiro-Wilk's test and Levene's *F*-test, respectively. Afterward, combined effects of seawater temperature and pH on the $\delta^{13}C$ values of seawater, soft tissue

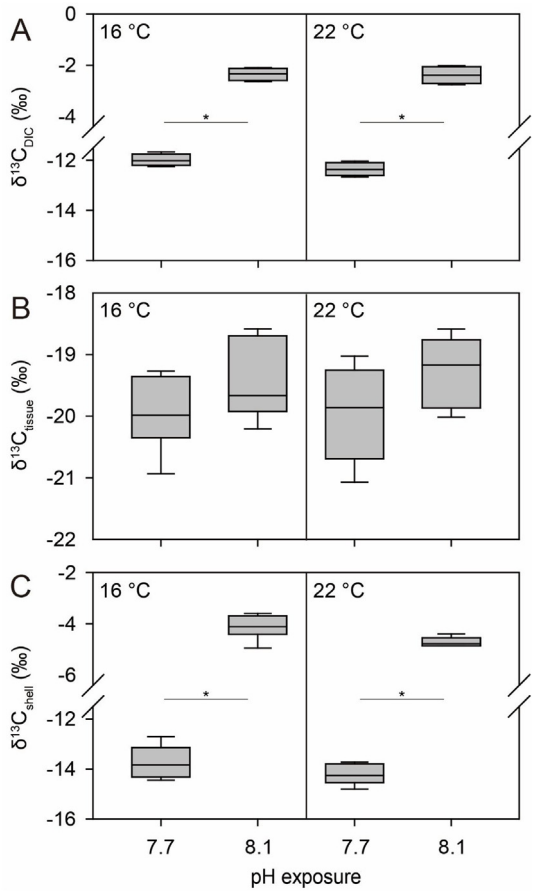

Fig. 3. Box plots showing $\delta^{13}C$ values of seawater DIC (a), *Mytilus edulis* soft tissues (b) and shells (c) under different temperature and pH scenarios. Asterisk indicates a significant effect of pH on $\delta^{13}C$ values at each temperature.

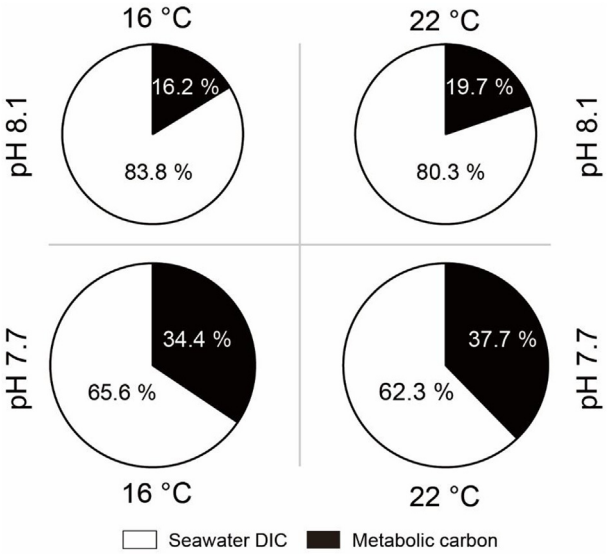

Fig. 4. Estimations of the percentage of metabolic carbon incorporation into *Mytilus edulis* shell carbonate reared at different temperature and pH scenarios.

and shell (n = 6) were evaluated using two-way analysis of variance (Two-way ANOVA). Significant difference was set at $p < 0.05$.

3. Results

Irrespective of temperature, which did not significantly affect the $\delta^{13}C_{DIC}$ of seawater ($p < 0.05$), bubbling highly ^{13}C-depleted CO_2 gas (Boutton, 1991) into the seawater resulted in a significant decrease of $\delta^{13}C_{DIC}$ ($p < 0.05$; Fig. 3a). On average, seawater $\delta^{13}C_{DIC}$ at pH 7.7 was 9.81‰ lighter than that at pH 8.1. Two-way ANOVA analysis showed that neither pH nor temperature significantly affected $\delta^{13}C$ values of mussel soft tissues ($p < 0.05$; Fig. 3b). In contrast, statistically significant effects of pH on $\delta^{13}C_{shell}$ were observed at both temperatures ($p < 0.05$; Fig. 3c). Mussel shells forming under acidified conditions (i.e., at pH 7.7) had $\delta^{13}C$ values approximately three times lower than those grown at pH 8.1.

As shown in Fig. 4, at pH 8.1, the percent metabolic carbon incorporation into mussel shell carbonate increased from 16.2% to 19.7% with increasing temperature from 16 to 22 °C. When exposed to reduced seawater pH, the percentage of metabolically generated carbon synthesized into mussel shell carbonate increased clearly to 34.4% at 16 °C and 37.7% at 22 °C, respectively.

4. Discussion

The present study demonstrates that up to 83.8% of *M. edulis* shell carbonate forming under normal conditions (i.e., at pH 8.1) originates from the DIC of ambient seawater. This finding agrees well with conclusions drawn in previous study (Gillikin et al., 2006) and many other bivalve species, for example *Ruditapes philippinarum* (Poulain et al., 2010), *Arctica islandica* (Beirne et al., 2012), *Pecten maximus* (Marchais et al., 2015) and *Perumytilus purpuratus* (Pérez et al., 2016).

As seawater temperature increases from 16 to 22 °C, irrespective of pH, the contribution of seawater DIC to *M. edulis* shell carbonate on average decreases by 3.4%, which, in turn, suggests an increasing uptake of metabolically generated CO_2 to the EPS. In mytilid mussels, increased filtering activity was reported to occur simultaneously with increases in temperature up to 24 °C (e.g., Anestis et al., 2010; Múgica et al., 2015; Wang et al., 2015). Our findings are in accordance with these observations, i.e., with increasing temperature mussels may likely consume more food at higher temperature, correspondingly respiring more and thus increasing the amount of metabolic CO_2 available for calcification. Yet, information is scarce regarding the temperature impact on the activity of molecular HCO_3^- transporters, hindering efforts to understand whether increases in calculated percent metabolic carbon contribution with increasing temperature are exclusively caused by increasing metabolic activities. Alternatively, as will be demonstrated below, elucidating the underlying mechanisms responsible for a large metabolic carbon incorporation under acidified conditions may fill this gap in knowledge.

Exposure of *M. edulis* to reduced seawater pH led to a significantly decreased percentage of seawater DIC synthesized into shell carbonate, irrespective of the fact that concentrations of HCO_3^- (the most calcification-relevant DIC species in seawater) increase with increasing pCO_2. Interestingly, this finding is rather comparable to observations by Zhao et al. (2018a), according to which the relative contribution of the seawater DIC taken up into the *R. philippinarum* shell carbonate decreased from 73% to 55% with reduced seawater pH from 8.1 to 7.7. Taken together, these results demonstrate that the ability of CO_2-exposed bivalves to efficiently transport seawater HCO_3^- for calcification is substantially affected. Evidence in support of this assumption also comes from the observations of decreased Na^+/HCO_3^- transporter and HCO_3^-/Cl^- exchanger activities in the outer mantle epithelium when subjected to reduced seawater pH (e.g., Alves and Oliveira, 2013; Li et al., 2016). In addition, the retention of metabolically generated CO_2 gas resulted from increasing seawater pCO_2 can likely constrain the passive diffusion of the latter into the EPS (Michaelidis et al., 2005; Zittier et al., 2015). On the other hand, ample evidence suggests that moderate seawater acidification may elicit an increase of metabolic activity in *M. edulis* (e.g., Thomsen et al., 2010; Sun et al., 2016; Mangan et al., 2017; Stapp et al., 2017), thus generating more CO_2

diffusing into the EPS.

In summary, the present study for the first time demonstrates increases in the contribution of metabolic carbon to the DIC pool at the calcifying front in the face of seawater acidification and warming. Metabolically generated CO_2 can easily and passively diffuse into the EPF. Thus, preferential uptake of metabolic carbon, which is much less energetically expensive than active transport of seawater DIC to the calcifying front, can potentially lessen the organismal energetic burden under acidified conditions. If this is the case, one would expect increases in the rate of shell formation as seawater pCO_2 and temperature increases. In contrast to this assumption, acidic exposure led to a significant decrease of mussel shell growth, as previously demonstrated by Zhao et al. (2017a). Evidently, such discrepancy can be most likely attributed to an important reaction in the EPS which has been largely ignored, i.e., whether and to what extent metabolically generated CO_2 can be quickly catalyzed to HCO_3^- by the carbonic anhydrase. Almost without exception, CO_2-exposed bivalves show consistently inhibited enzymatic activities (e.g., Ivanina et al., 2013; Moreira et al., 2016; Wang et al., 2017; Jeffrey et al., 2018), accordingly resulting in the retention of CO_2 (and hence protons) to a certain degree in the calcifying fluid which have been previously demonstrated by Zhao et al. (2017a). As such, a causal link between the percentage of metabolic carbon synthesized into mussel shell carbonate and the rate of shell calcification (the latter is closely linked to the rate of shell growth) was not observed in the present study. Given the energetically expensive nature of shell formation, much more effort should be devoted to gaining a better understanding of how near-future scenarios of ocean acidification and warming affect the overall energy balance and the energy partitioning and reallocation in differential mineralization processes such as (1) the uptake of seawater DIC to the calcifying front, (2) the maintenance of the acid-base homeostasis in the calcifying fluid and (3) the synthesis and excretion of organic matrix utilized for $CaCO_3$ crystallization.

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